



Protein hydrolysates from *Tenebrio molitor* and *Hermetia illucens* as novel food sources for dogs

D. Leal,[†] S. Borges,[‡] A. Almeida,[‡] M. Pintado,[‡] A.A.J.M. Fonseca,^{†,1}  and A.R.J. Cabrita^{†,1} 

[†]LAQV, REQUIMTE, ICBAS, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, 4050-313, Portugal

[‡]Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina - Laboratório Associado, Escola Superior de Biotecnologia, Porto, 4169-005, Portugal

¹ETSA, SGPS, S.A, Loures, 2660-119, Portugal

¹Corresponding author: arcabrita@icbas.up.pt

ABSTRACT

There has been a growing interest in insect meals as a sustainable alternative protein source for animal food and feed. In parallel, insect hydrolysates have been investigated in vitro for their bioactive properties, but the impact of dietary inclusion on dog's nutritional parameters needs to be elucidated. This study aimed to evaluate the chemical composition and bioactive properties of four insect hydrolysates obtained from the enzymatic hydrolysis of *Tenebrio molitor* and *Hermetia illucens* using ALCALASE 2.5L and Corolase PP enzymes and investigate the effects of their dietary inclusion on diet palatability, digestibility, and fecal characteristics and metabolites of adult Beagle dogs. Four two-bowl tests were performed using 12 adult Beagle dogs to assess palatability by the pairwise comparison between the control diet (a commercial diet with 3% of shrimp hydrolysate) and each of the four experimental diets (control diet with the replacement of 3% (w/w) of shrimp hydrolysate by 3% of each insect protein hydrolysate). A digestibility trial designed according to a replicated Latin square 5 × 5 design, with ten adult dogs, five periods of 10 d each, and five diets was performed to evaluate the effects on food intake, fecal characteristics and metabolites, apparent total tract digestibility, and estimated metabolizable energy content of the control and experimental diets. Chemical composition and in vitro antioxidant and antihypertensive activities of insect hydrolysates depended on the insect species and enzyme used. No differences were observed in the first diet approached or tasted, but the inclusion of hydrolysates of *T. molitor* obtained from Corolase PP hydrolysis showed a greater intake ratio ($P = 0.032$). Food intake, diet digestibility and fecal characteristics were not different between diets, except for an increased fecal caproate concentration in dogs fed the control diet ($P = 0.024$). The dietary inclusion of insect hydrolysates did not affect nutritional parameters, and further investigation is needed to evaluate their health-promoting properties for pet foods and supplements.

Lay Summary

Dog owners are increasingly seeking more sustainable foods and supplements with high nutritional and functional values to promote the overall well-being, longevity, and quality of life of their companion animals. Insects such as black soldier fly (BSF) and yellow mealworm (YM) comprise available protein sources with a sustainable potential and bioactive properties. However, in-depth research studies on the benefits of dietary inclusion of insect hydrolysates are still needed. This study evaluated the chemical composition and in vitro antihypertensive and antioxidant activities of four insect hydrolysates obtained from the enzymatic hydrolysis of BSF and YM, as well as the effects of their dietary inclusion on diet palatability, digestibility and fecal characteristics, and metabolites of healthy adult Beagle dogs. Despite all hydrolysates having been shown to have high protein content and bioactive properties, results differed between insect species and enzymes used. Inclusion of insect hydrolysates (3% w/w) in substitution of shrimp hydrolysate in a complete diet showed to be well accepted by dogs, maintaining diet digestibility, fecal characteristics and metabolites. Overall, results showed that insect protein hydrolysates could be explored as new food sources for dog nutrition.

Key words: black soldier fly, dog nutrition, insect hydrolysates, yellow mealworm

INTRODUCTION

The global population of dogs and cats has been increasing, with the recent FEDIAF (European Pet Food Industry Federation) annual report (FEDIAF, 2024a) showing that, in 2022, 166 million European households (50%) own one or more of Europe's 352 million pets. Consequently, the pet food industry is continuously growing, challenging its sustainability and environmental impact (Alexander et al., 2020). Simultaneously, dog owners are increasingly demanding foods and supplements with high nutritive and functional values to promote the overall well-being, longevity, and quality of life of their companion animals (Okin, 2017; Pedrinelli et al., 2022). Therefore, it is imperative to find innovative approaches to

reduce the competition for food resources for both human and animal feeding, while promoting sustainability and benefiting animal health (Oonincx and de Boer, 2012).

Insects are a newly available protein source for pet food products with a sustainable potential, presenting a high feed efficiency, with lower requirements for water, land and feed, compared to traditional protein sources (e.g., chicken, beef, turkey, and salmon; Oonincx and de Boer, 2012; Bosch et al., 2014). Currently, the global production of insect meals is primarily used for pet food and aquaculture feed, with black soldier fly (BSF, *Hermetia illucens*) and yellow mealworm (YM, *Tenebrio molitor*) being the two most commercially used edible insect species (Mancini et al., 2022). The capability

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to regulate the life cycle of BSF and YM, including their mass rearing and efficient protein production on minimal land area, as well as the bioconversion of waste materials, facilitates large-scale and sustainable production (Hancz et al., 2024). Moreover, BSF and YM meals have a high protein content (approximately 40% to 60%), are rich in essential amino acids (EAA) like leucine, lysine and valine, and are also abundant in linoleic acid and functional peptides, offering bioactive properties that make them ingredients with a good overall nutrient profile to be used in pet food (Bosch et al., 2014; Bosch and Swanson, 2021; Ordoñez-Araque et al., 2022). However, studies on pet owner's acceptance indicate customers are divided when it comes to paying for insect-based pet foods (Fantechi et al., 2024). There are already published studies with dogs showing the effects of the dietary inclusion of BSF meals on palatability and digestibility, demonstrating promising results, with good diet acceptance and nutrient digestibility (Abd El-Wahab et al., 2021; Freel et al., 2021; Penazzi et al., 2021), but in-depth research studies on the benefits of dietary inclusion of insect hydrolysates are still needed. In vitro studies have shown that insect-derived hydrolysates are particularly rich in functional peptides, possessing antioxidant, antihypertensive, anti-inflammatory, and immunomodulatory effects (Tang et al., 2018; Batish et al., 2020; Brai et al., 2022; Aiello et al., 2023). These properties can bring functionality to pet foods and supplements, with a promising potential to prevent and help manage some of the most common chronic degenerative diseases in dogs, like osteoarthritis, neoplastic or cardiac diseases (Mouithys-Mickalad et al., 2021; Nowakowski et al., 2022). As seen for other protein hydrolysates obtained from animal products (Martínez-Alvarez et al., 2015; Romero-Garay et al., 2022; Szczepanik et al., 2022), hydrolysis of animal proteins brings functional properties and can act as food palatability enhancers. In addition to this potential, insect hydrolysates can be used as a novel ingredient to create hypoallergenic foods, addressing a common issue related to food allergies and digestive sensitivities in dogs (Simpson et al., 2023). However, as mentioned earlier, the potential of insect hydrolysates for pet food is still almost unknown. Indeed, to the best of the author's knowledge, only one in vivo study addressed the effects of the dietary inclusion of a mixture of BSF larvae protein hydrolysate (from 5% to 15% of dietary inclusion) combined with *Schizochytrium* on the health parameters of Beagle dogs. Despite showing significant enhancements in palatability, immune modulation, antioxidant activity, anti-inflammatory responses, and a marked reduction in diarrhea incidence, it is not possible to distinguish the observed effects of BSF hydrolysate from those of *Schizochytrium* (Wei et al., 2024).

To unveil the potential of insect hydrolysates for dog feeding, the present study aimed to evaluate the chemical composition and in vitro antihypertensive and antioxidant activities of four insect hydrolysates obtained from the enzymatic hydrolysis of BSF and YM, as well as investigate their effects on diet palatability, digestibility, and fecal characteristics and metabolites of healthy adult Beagle dogs.

MATERIALS AND METHODS

Trials were approved by the Animal Ethics Committee of School of Medicine and Biomedical Sciences, University of Porto (Permit N° 430) and conducted after good animal

welfare practices (European Union Directive 2010/63/EU) by trained scientists (FELASA, category C). Before starting the trials, all dogs underwent a complete physical examination at the Veterinary Hospital of School of Medicine and Biomedical Sciences, University of Porto, including the assessment of heart and respiratory rates, body temperature, mucous membrane color, capillary refill time, auscultation of the heart and lungs, abdominal palpation, lymph node palpation, and evaluation of hydration status.

Animals and Housing

Twelve healthy Beagle dogs, 6 males and 6 females, 5 ± 0.4 yr old, 12.8 ± 2.36 kg body weight (BW), 5.0 ± 0.30 out of 9 body condition score (BCS; Freeman et al., 2011), were used in the palatability trial. Then, 10 healthy Beagle dogs, 5 males and 5 females, 5 ± 0.4 yr old, 13.2 ± 2.27 kg BW, BCS 5.0 ± 0.28 , were randomly selected from the initial group of twelve dogs to participate in the digestibility trial. The number of animals followed the recommendations for minimum sample size number (FEDIAF, 2024b). Animals were housed in pairs, at the kennel of the School of Medicine and Biomedical Sciences, University of Porto, in communicating boxes with interior and exterior areas of 1.8 and 3.5 m², respectively. Animals had free access to an outdoor park area between daily meals to exercise and socialize and were leash-walked once a day for at least 30 min. During the total feces collection period, dogs were housed individually at night, having supervised access to the outdoor park between daily meals and daily leash walking.

Hydrolysates and Experimental Diets

Insect hydrolysates were provided by ETSA-SGPS, S.A. (Loures, Portugal) and were obtained after enzymatic hydrolysis of two insect larvae species (BSF and YM), using different commercial enzymes (ALCALASE 2.5L and Corolase PP). BSF and YM were provided by Hermetia Baruth GmbH (Baruth, Germany) and TecmaFoods (Leça do Balio, Portugal), respectively, and ALCALASE 2.5L and Corolase PP were supplied by Univar Solutions Inc. (Illinois, USA) and AB Enzymes (Darmstadt, Germany), respectively. The hydrolysates were prepared from insect meals defatted by the producer. Enzymatic hydrolysis with ALCALASE 2.5L was performed using 1% enzyme/substrate ratio (w/w) during 4 h at 65 °C, while enzymatic hydrolysis with Corolase PP was performed using 2% enzyme/substrate ratio (w/w) during 4 h at 60 °C. After enzymatic hydrolysis, the enzymes were inactivated by heating the mixture at 90 °C for 10 to 15 min. The hydrolysates were then filtered through a 450 µm sieve to remove solid materials, followed by centrifugation at 5394 x g at room temperature to separate solids, lipids, and proteins. The protein fraction was isolated using a separatory funnel, concentrated using a rotary evaporator, and dried using a Buchi B-290 Spray Dryer (Buchi, Switzerland). Shrimp hydrolysate was provided by Symrise Aqua Feed (Elven, France) and resulted from the enzymatic hydrolysis of heads and cephalothoraxes of *Litopenaeus vannamei*, with solid waste being separated by centrifugation at the end of the hydrolysis process. As all hydrolysates were obtained from the soluble fraction following enzymatic hydrolysis, insoluble components, such as chitin, are expected to be present in residual amounts. All protein hydrolysates were provided as a dry powder and kept at room temperature until use.

A commercial extruded complete diet for adult medium-size dogs (Sorgal Pet Food, Ovar, Portugal) with the addition of 3% (w/w) of shrimp hydrolysate in replacement of the basal diet was used as the control diet. Four experimental diets were prepared with the same base commercial diet except for the replacement of shrimp hydrolysate by 3% (w/w) of BSF hydrolyzed with ALCALASE 2.5L (BSF-A) or Corolase PP (BSF-C) or by YM hydrolyzed with ALCALASE 2.5L (YM-A) or Corolase PP (YM-C). The hydrolysates were added as dry powders directly to each dog's bowl on top of the extruded kibbles and thoroughly mixed immediately before feeding. Visual appraisal of each bowl at each meal confirmed that the dose of hydrolysate provided was completely ingested.

Palatability

Four two-bowl tests were conducted to determine palatability by the pairwise comparison of the control diet with the diets containing insect hydrolysates (Aldrich and Koppel, 2015). On two consecutive days, animals ($n = 12$) were given the choice between two diets in two different bowls (45 cm apart) in both daily meals, with the position of the bowls being switched between meals to control side bias. Each bowl contained the amount of food calculated to meet the metabolizable energy (ME) requirements for each meal, with dogs thus being offered twice their ME requirements (FEDIAF, 2024b). Trials ended after 30 min or when the total amount of food offered in one bowl was consumed. The bowl that was first approached and the first food tasted were registered and the consumption amount of each diet was recorded to calculate the intake ratio of the two diets.

Apparent Total Tract Digestibility

The total feces collection method was used to determine the apparent total tract digestibility (ATTD) of control and experimental diets. The trial was designed according to a replicated Latin square 5×5 , with 5 diets, 10 adult Beagle dogs (5 males and 5 females), and 5 experimental periods of 10 d each, with 5 d for adaptation and 5 d for total feces collection. Daily food allowance was determined to ensure the daily requirements of ME according to the BW of the animals calculated as $110 \times \text{BW}^{0.75}$ kcal (FEDIAF, 2024b), and adjusted to the ideal BCS (Freeman et al., 2011). Daily food was individually distributed to each animal in two equal meals (08:30h and 16:30h). Animals had free access to fresh water at all times. During the total feces collection periods, the number of defecations was recorded, and individual samples were immediately collected after defecation, weighed, scored using a 5-point scale to evaluate the consistency of stools (where 1 stands for watery feces and 5 for hard feces; Carciofi et al., 2008), and stored at -20°C for further analysis. At the end of each experimental period, dogs were weighed and their BCS was assessed before the morning meal.

Chemical Analysis

The base diet and the hydrolysates were dried in an air-forced oven at 65°C until constant weight, the base diet ground (1 mm) and all analyzed according to official methods (AOAC, 2005). Briefly, samples were analyzed for dry matter (DM; ID 934.01), ash (ID 942.05), Kjeldahl N (ID 990.03) and ether extract (EE; ID 920.39). Crude protein (CP) was calculated as Kjeldahl N $\times 6.25$. Starch was analyzed as described by Salomonsson et al. (1984). Gross energy (GE) analysis was performed with an adiabatic bomb calorimeter (Werke C2000,

IKA, Staufen, Germany). The base diet was also analyzed for neutral detergent fiber (NDF, with α -amylase and without sodium sulphite, expressed exclusive of residual ash; Van Soest et al., 1991). The amino acid (AA) profile of the base diet and the hydrolysates was analyzed through iodoacetic acid derivatization and o-phthalaldehyde assay. Briefly, reverse phase high-performance liquid chromatography (RP-HPLC, Beckman Coulter, USA) was utilized for chromatographic separation (Oliveira et al., 2022). An acid hydrolysis was performed according to Wang et al. (2016), and free AA were quantified according to Pripis-Nicolau et al. (2001), by using calibration curves of pure standard AA. To minimize the oxidation of sulfur-containing AA, particularly methionine and cysteine, tubes were flushed with nitrogen during acid hydrolysis. Feces were thawed at room temperature for a short period of time (up to 15 min), manually homogenized using a spatula for approximately 30 s, and representative samples obtained through collection at multiple points were immediately analyzed for N (in fresh fecal samples, with CP calculated as $\text{N} \times 6.25$), and ash, EE, NDF, starch (0.5-mm milled) and GE contents were analyzed on dried (65°C) and milled (1-mm) feces pooled per dog and period. The analysis were performed according to the methods described above for diet samples.

All analyses were performed in duplicate.

Fecal End-Fermentation Products

The pH of thawed and homogenized fecal samples was measured using a potentiometer (sensION + PH3, Hach-Langue, S.L.U., Barcelona, Spain). The concentration of fecal ammonia-N was determined as described by Chaney and Marbach (1962). First, thawed feces were diluted to 1:10 (w/v) in 2 M KCl and centrifuged (12,000 g, 4°C , 20 min). The supernatant was filtered using a $0.45\ \mu\text{m}$ pore size polyethersulfone syringe filter (FILTER-LAB, Barcelona, Spain) and 40 μL of water was added to 40 μL of sample, followed by the addition of 2.5 mL of phenol solution and 2 mL of 0.37% alkaline hypochlorite solution. Samples were incubated for 10 min at 37°C , followed by 40 min of incubation at 22°C in the dark, and absorbance was read at 550 nm in a SYNERGY HT Multimode plate reader (BIOTEK Instruments Inc., Winooski, VT, USA). An ammonia solution (32 mg/dL) was used as a standard and analysis was done in duplicate. Short-chain fatty acids (SCFA) concentrations were analyzed by gas chromatography as described by Pereira et al. (2021). One g of feces was diluted in 10 mL of 25% orthophosphoric acid solution with an internal standard (4 mM 3-methyl valerate, Sigma-Aldrich, Saint Louis, MO, USA), centrifuged for 16 min at 18,000 g at 4°C , and the supernatant was stored at -20°C until analysis. Values were expressed on a DM basis. Analyses were performed in duplicate.

In Vitro Antihypertensive and Antioxidant Activities of Hydrolysates

To assess the in vitro antihypertensive activity of the hydrolysates, the inhibitory effect of angiotensin-converting enzyme (ACE) was measured by the fluorometric assay proposed by Sentandreu and Toldra (2006) and modified according to Quirós et al. (2009). Briefly, o-Abz-Gly-p-Phe(NO₂)-Pro-OH 0.45 mM (Bachem, Switzerland) was used as a substrate and the reaction was carried out in 0.04 U/mL of ACE (peptidyl-dipeptidase A, EC 3.4.15.1), pH 8.3 with 0.1 mM ZnCl_2 (Sigma-Aldrich, Missouri, USA).

The reaction mixture was incubated at 37 °C and fluorescence was measured after 30 min using a Multi Detection plate reader (Synergy H1, Vermont, USA). The wavelengths used were 350 nm (excitation) and 420 nm (emission). The ACE inhibitory activity was analyzed in triplicate for each sample by calculating the half-maximal inhibitory concentration (IC_{50}), which determines the protein hydrolysate concentration needed to inhibit 50% of ACE activity. The total antioxidant activity of hydrolysates was determined by ABTS radical scavenging activity (Re et al., 1999) and by the oxygen radical absorbance capacity (ORAC) methodology (Contreras et al., 2011), with final values being expressed as Trolox equivalent (TE) $\mu\text{mol/g}$. The interaction of 2.45 mM potassium persulfate (Sigma-Aldrich, Saint Louis, MO, USA) and 7 mM 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, Saint Louis, MO, USA) yielded ABTS radical cation (ABTS $\bullet+$). Then, the reaction was measured at 734 nm using 180 μL of ABTS $\bullet+$ with 20 μL of sample or Trolox as the standard calibration curve (25 to 175 M). All reactions were performed in triplicate. For ORAC, samples were dissolved in 75 mM phosphate buffer (pH 7.4) and the solution was mixed with 120 μL of fluorescein (70 nM) (Sigma-Aldrich, Saint Louis, MO, USA) and incubated at 37 °C for 10 min. Then, 60 μL of 2,20-Azobis(2-amidinopropane) dihydrochloride solution (14 mM; Sigma-Aldrich, Saint Louis, MO, USA) was added to the mixture. The fluorescence was recorded for 140 min using a Multidetector plate reader (Synergy H1, Vermont, USA) at excitation and emission wavelengths of 485 and 528 nm, respectively. All reactions were performed in duplicate.

Calculations and Statistical Analysis

The ATTD was calculated according to:

$$\text{ATTD (\%)} = \frac{g \text{ nutrient intake/d} - g \text{ fecal nutrient/d}}{g \text{ nutrient intake/d}} \times 100.$$

The ME content of diets was estimated according to Hall et al. (2013):

$$\text{ME (MJ/kg)} = \frac{((kJ \text{ GE intake/d} - kJ \text{ fecal GE/d}) - [g \text{ CP intake/d} - g \text{ fecal CP/d}] \times 5.23)}{g \text{ DM intake/d}}$$

The chemical composition of diets offered to dogs was calculated from the analyzed chemical composition of basal diet and each hydrolysate. All data were analyzed using SAS software (2022, release 3.81., SAS Institute Inc., Cary, NC, USA). Diet first-approach and first-taste results were submitted to the Chi-square test and the intake ratio to the Student's t-test. Data from the digestibility trial was analyzed using the general linear model with the fixed effects of the square, dog within the square, period, diet, and residual error. When differences were significant ($P < 0.05$), Tukey's test was used to compare means. Data on fecal consistency was analyzed using a Wilcoxon rank sum test to compare scores between dogs fed different diets, according to Scheff (2016).

RESULTS

Chemical Composition of Hydrolysates and Experimental Diets

The proximate chemical composition and AA content of hydrolysates are presented in Table 1. Compared to shrimp

hydrolysate, insect hydrolysates presented lower CP and EE contents. The CP content of insect hydrolysates ranged from 58.0 in BSF-C to 66.6% (DM basis) in BSF-A. Similarly, BSF-C presented the lowest total AA content and BSF-A the greatest content, among insect hydrolysates. Arginine, leucine and lysine were the EAA presented in greater amounts in shrimp hydrolysate, whereas leucine, valine and tyrosine were those found in greater amounts in insect hydrolysates, with lysine being also presented in greater amounts in YM-C and YM-A. The inclusion of 3% (w/w) of each hydrolysate at the expense of the base diet did not impact the chemical composition of diets (Table 2).

In Vitro Antioxidant and Antihypertensive Activities of Hydrolysates

The in vitro antioxidant and antihypertensive activities of hydrolysates studied are outlined in Table 3. Insect hydrolysates obtained through ALCALASE 2.5L hydrolysis demonstrated greater antioxidant activity on both ABTS and ORAC methods than hydrolysis with Corolase PP. With the ABTS method, shrimp hydrolysate exhibited similar antioxidant activity to BSF-A, YM-C and YM-A, whereas with the ORAC method presented a lower antioxidant activity compared to the same insect hydrolysates. All hydrolysates demonstrated antihypertensive activity, with YM-C having the lowest IC_{50} , thus reflecting a higher in vitro antihypertensive activity. Compared to insect hydrolysates, shrimp hydrolysate presented the lowest antihypertensive activity.

Palatability

The results of the four two-bowl palatability tests are presented in Table 4. No differences were observed on first diet approached or tasted, but dogs showed a preference ($P = 0.032$) for the diet supplemented with YM-C (59% intake ratio) when compared to the control diet, without significant differences on the intake ratio for the diets containing other hydrolysates.

Body Weight, Body Condition Score, Diet and Nutrient Intake

Dogs remained healthy throughout the feeding trial, with a stable BCS and BW, and without food refusals or episodes of emesis or diarrhea across diets. The median BCS of dogs during the study was 5 out of 9 for experimental and control diets. No significant difference ($P = 0.741$) was observed on food intake among diets that ranged from 211 to 216 g/d with diet supplemented with YM-C and shrimp hydrolysate, respectively.

Fecal Output, Characteristics and Metabolites

The dietary inclusion of insect hydrolysates kept the fecal consistency unaffected, with fecal scores of 3.5 out of 5 for all diets ($P > 0.05$; Figure 1). No differences were observed in fecal output, pH, ammonia-N concentration and concentrations of SCFA, except for a greater concentration of fecal caproate in dogs fed the control diet with shrimp hydrolysate compared to those eating diets containing YM hydrolysates ($P = 0.024$; Table 5).

Apparent Total Tract Digestibility and Metabolizable Energy

The inclusion of insect hydrolysates kept ATTD and ME content of diets unaffected (Table 6), with no significant differences compared to the control diet.

Table 1. Proximate chemical composition and amino acid (AA) content of hydrolysates expressed as g/100 g of dry matter

	Protein Hydrolysates ¹				
	Shrimp	BSF-C	BSF-A	YM-C	YM-A
Dry matter, as is	96.2	94.5	95.8	95.4	96.0
Ash	12.5	14.7	10.1	9.01	8.70
Crude protein	70.1	58.0	66.6	64.7	62.4
Ether extract	8.85	2.29	1.62	3.52	1.90
Starch	0.83	10.59	9.45	6.46	10.66
Gross energy, MJ/kg	20.9	18.4	19.4	20.3	20.4
Essential AA (EAA)					
Arginine	4.45	2.03	3.31	2.43	2.43
Histidine	1.42	1.70	1.96	1.73	1.83
Lysine	4.29	2.55	3.45	3.56	3.71
Threonine	2.66	2.03	2.74	2.50	2.66
Isoleucine	3.03	2.13	2.87	2.57	2.78
Leucine	4.39	2.63	4.00	3.82	4.20
Valine	3.37	2.85	4.03	3.52	3.92
Methionine	1.44	0.88	0.90	0.89	0.94
Phenylalanine	3.20	1.73	2.45	1.97	2.07
Cysteine	0.87	0.85	0.88	0.86	0.90
Tyrosine	2.85	2.70	4.16	3.61	3.72
Total EAA	32.0	22.1	30.8	27.4	29.2
Non-essential AA (NEAA)					
Aspartic acid + Asparagine	5.81	4.28	5.37	5.12	5.09
Glutamic acid + Glutamine	8.66	7.57	7.64	8.46	8.73
Alanine	3.86	4.52	5.23	4.35	4.63
Glycine	4.83	2.76	3.44	2.90	3.18
Proline	3.71	3.55	4.22	4.38	5.73
Serine	2.68	2.23	3.13	2.73	2.75
Taurine	1.09	0.07	0.07	0.12	0.11
Total NEAA	30.6	25.0	29.1	28.1	30.2
Total AA	62.6	47.1	59.9	55.5	59.4

¹BSF-C, black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; BSF-A, black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; YM-C, yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; YM-A, yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L.

DISCUSSION

Protein hydrolysates hold great potential for various applications in pet food, with chicken, chicken liver, and fish being the most commonly used sources to produce such hydrolysates (Hou et al., 2017; Sánchez-Estrada et al., 2024). They can be included as an ingredient, with an inclusion of 15% to 25%, with lower allergenicity and greater digestibility compared to non-hydrolyzed sources, or as a supplement at 0.01% to 6% with functional properties or as an attractant (Samant et al., 2021; Hsu et al., 2024a, 2024b). The present study provides a first assessment of in vitro antihypertensive and antioxidant properties of insect hydrolysates and their effects when included as functional ingredients in diets for adult dogs. Although chicken and fish hydrolysates are more commonly used in commercial diets, thus being already extensively studied, in the present study, diets containing insect hydrolysates were tested in comparison to a diet containing shrimp hydrolysate, derived from processing byproducts (heads and exoskeletons). This approach is justified by the recent evaluation of shrimp hydrolysate in short and long-term in vivo trials that showed to be well accepted by dogs,

presenting high digestibility, antioxidant and prebiotic effects, as well as health benefits (Guilherme-Fernandes et al., 2024a,b, 2025).

Chemical Composition of Hydrolysates and Diets

The four insect hydrolysates analyzed in the present study exhibited lower CP and EE contents than shrimp hydrolysate, whose composition agrees with earlier studies (Latorres et al., 2018; Guilherme-Fernandes et al., 2024a, 2024b). In comparison to other common protein hydrolysates derived from fish or chicken, which exhibit protein contents ranging from 60% to 82% (DM basis; Aspevik et al., 2021; Ryu et al., 2021; Wu et al., 2022), the insect hydrolysates analyzed demonstrated a similar to lower protein content, ranging from 58% to 67% (DM basis). Among insect hydrolysates, BSF and YM treated with ALCALASE 2.5L presented a greater total AA content and lower EE content compared to those treated with Corolase PP, highlighting the impact of enzymatic hydrolysis methods on the chemical composition.

Leucine, valine and tyrosine were the EAA found in greater amounts in insect hydrolysates. Branched-chain EAA, such

Table 2. Proximate chemical composition and amino acid (AA) profile of diets containing hydrolysates fed to dogs during the palatability and digestibility trials expressed as g/100 g of dry matter¹

Composition	Diets ²				
	D_Shrimp	D_BSF-C	D_BSF-A	D_YM-C	D_YM-A
Dry matter, as is	94.9	94.9	94.9	94.9	94.9
Ash	7.92	7.99	7.85	7.82	7.81
Crude protein	29.4	29.1	29.3	29.3	29.2
Ether extract	11.4	11.2	11.2	11.2	11.2
NDF	14.4	14.4	14.4	14.4	14.4
Starch	33.4	33.7	33.7	33.6	33.7
Gross energy, MJ/kg	20.8	20.7	20.7	20.8	20.8
Essential AA (EAA)					
Arginine	1.81	1.74	1.78	1.75	1.75
Histidine	0.66	0.67	0.68	0.67	0.67
Lysine	1.57	1.52	1.54	1.55	1.55
Threonine	1.21	1.19	1.22	1.21	1.21
Isoleucine	1.47	1.44	1.47	1.46	1.46
Leucine	2.39	2.33	2.37	2.37	2.38
Valine	1.55	1.53	1.57	1.55	1.56
Methionine	0.86	0.84	0.84	0.84	0.85
Phenylalanine	1.65	1.61	1.63	1.62	1.62
Cysteine	0.87	0.87	0.87	0.87	0.87
Tyrosine	1.60	1.60	1.64	1.62	1.63
Total EAA	15.7	15.4	15.6	15.5	15.6
Non-essential AA (NEAA)					
Aspartic acid + Asparagine	2.34	2.29	2.32	2.32	2.32
Glutamic acid + Glutamine	4.28	4.25	4.25	4.28	4.28
Alanine	1.75	1.77	1.79	1.76	1.77
Glycine	1.94	1.88	1.90	1.88	1.89
Proline	1.92	1.92	1.94	1.94	1.98
Serine	1.43	1.41	1.44	1.43	1.43
Taurine	0.17	0.14	0.14	0.15	0.15
Total NEAA	13.8	13.7	13.8	13.8	13.8
Total AA	29.5	29.1	29.4	29.3	29.4

¹Calculated from the analyzed chemical composition of the basal diet and each protein hydrolysate.

²D_Shrimp, complete diet with shrimp hydrolysate; D_BSF-C, complete diet with black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; D_BSF-A, complete diet with black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; D_YM-C, complete diet with yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; D_YM-A, complete diet with yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L.

Table 3. In vitro antioxidant activity (ABTS and ORAC methods) and antihypertensive activity (IC₅₀) of hydrolysates obtained through enzymatic hydrolysis

In vitro functional activities	Protein hydrolysates ¹				
	Shrimp	BSF-C	BSF-A	YM-C	YM-A
ABTS, μmol TE ³ /g hydrolysate	389 ± 2.5	254 ± 3.2	337 ± 19.4	354 ± 10.7	393 ± 20.3
ORAC, μmol TE/g hydrolysate	530 ± 40.8	488 ± 53.9	787 ± 39.4	624 ± 49.5	741 ± 26.9
IC ₅₀ ² , mg hydrolysate/mL	2.3 ± 0.04	1.2 ± 0.01	1.0 ± 0.01	0.6 ± 0.01	1.3 ± 0.02

¹BSF-C, black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; BSF-A, black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; YM-C, yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; YM-A, yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L; ²IC₅₀, lower half-maximal inhibitory concentration; ³TE, Trolox equivalent.

as valine and leucine, are involved in muscle formation and hepatic regeneration and might contribute to scavenge or inhibit free radicals through indirect stimulation of glutathione production, thus having an important role in the

antioxidant capacity (Saadi et al., 2015; Zhou et al., 2022). Glutamic and aspartic acids were the most abundant AA found in the insect hydrolysates and, although they are non-essential for dogs, their inclusion in the diet can positively

Table 4. Diet first approached and first tasted frequency and intake ratio of diets containing insect hydrolysates compared to a control diet containing shrimp hydrolysate

Item	Diets ¹			
	D_BSF-A	P-value	D_BSF-C	P-value
First approach	50%	1.000	48%	0.773
First taste	50%	1.000	48%	0.648
Intake ratio	48%	0.601	46%	0.415
Item	Diets ¹			
	D_YM-A	P-value	D_YM-C	P-value
First approach	56%	0.386	48%	0.773
First taste	50%	1.000	56%	0.386
Intake ratio	41%	0.130	59%	0.032

¹D_Shrimp, complete diet with shrimp hydrolysate; D_BSF-C, complete diet with black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; D_BSF-A, complete diet with black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; D_YM-C, complete diet with yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; D_YM-A, complete diet with yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L.

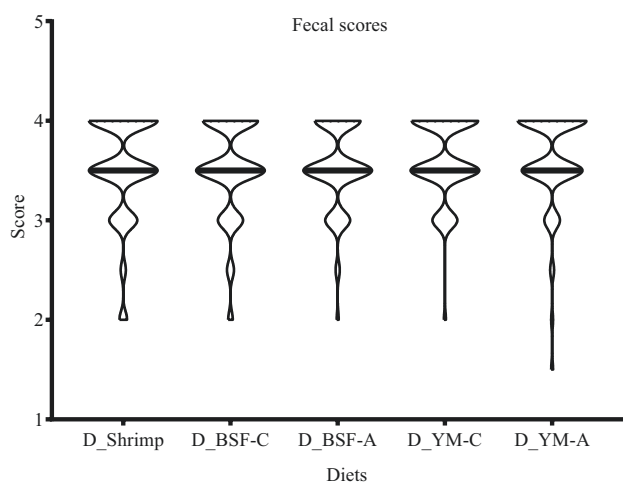


Figure 1. Fecal scores of dogs fed diets containing hydrolysates. The violin plot illustrates the distribution of the scores. Wider sections of the plot indicate a higher concentration of data points, and narrower sections indicate fewer data points. The median scores are represented by the line within the plot ($P > 0.05$). D_Shrimp, complete diet with shrimp hydrolysate; D_BSF-C, complete diet with black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; D_BSF-A, complete diet with black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; D_YM-C, complete diet with yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; D_YM-A, complete diet with yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L.

impact intestinal metabolism, with glutamic acid, in particular, being recognized also for its role as a flavor enhancer (Li and Wu, 2023). Previous studies reported leucine, lysine and valine as the most abundant EAA in BSF hydrolysates (Firmansyah and Abduh, 2019; Batish et al., 2020; Zhu et al., 2020). Regarding YM hydrolysates, Tang et al. (2018) observed lower total AA content in YM-A compared to our results. The differences in chemical composition observed among published studies might reflect different insect production methods and hydrolysis conditions (López-Gómez et al., 2024).

The low inclusion level (3%) of hydrolysates kept unaffected the chemical composition of diets that ensured the minimum recommended levels of nutrients and energy for adult dogs (FEDIAF, 2024b).

In Vitro Antioxidant and Antihypertensive Properties of Hydrolysates

Bioactive peptides are short sequences of 2 to 30 AA that exhibit biological activity beyond their nutritional value. Their bioactivity and bioavailability depend on the electrostatic charge, chain size, AA sequences in the peptide chain, molecular surface hydrophobicity, and the stability of peptides in gastrointestinal digestion (Xue et al., 2021; Vasconcellos et al., 2024). Despite both enzymes (ALCALASE 2.5L and Corolase PP) being endopeptidases, they present different mechanisms of action. ALCALASE 2.5L preferably cleaves proteins in areas with reduced hydrophilicity, and accessible to the enzyme, commonly associated with aromatic AA (Evans et al., 2000). Studies have suggested leucine, valine, isoleucine, phenylalanine, tryptophan and tyrosine as preferred, but not exclusive, to precede the cleavage site (Evans et al., 2000). However, the hydrophobicity of the adjacent residues plays a major role in the definition of the cleavage site (Adamson and Reynolds, 1996). Peptides formed from the hydrolysis using Alcalase vary widely but are enriched in peptides with less than 1KDa, creating very small peptides enriched in hydrophobic residues in the extremities (Adamson and Reynolds, 1996; Chewaka et al., 2023). Corolase PP is known to preferably cleave proteins after nonpolar AA, such as alanine, without following a very rigid sequence for cleavage (Peterle et al., 2020). The peptides formed after Corolase PP 7089 hydrolysis usually range between 0.8 to 10KDa (Conti et al., 2019), leading to the formation of several different peptides with possible bioactivities. Considering the cleaving mechanisms for both enzyme types and their low specificity, it is expected to obtain hydrolysates with low molecular weight peptides, increasing the possibility of developing bioactivities, and with different properties, depending on which enzyme was used.

Several in vitro bioactive properties are described for functional peptides obtained from insect hydrolysates, including antioxidant, antihypertensive, anti-inflammatory, antidiabetic, immunomodulatory, and antimicrobial activities (Nongonierma and FitzGerald, 2017; Hasnan et al., 2023; Teixeira et al., 2023). In the present study, in vitro antioxidant activity was evaluated by the ABTS and ORAC methods. The ABTS assay measures the relative ability of antioxidants to scavenge ABTS-generated radicals compared with a Trolox standard and can be used to evaluate both lipophilic and hydrophilic materials. In contrast, the ORAC method may not

Table 5. Fecal output, pH, and concentration of ammonia-N (g/kg, dry matter, DM) and short-chain fatty acids (SCFA; $\mu\text{mol/g}$, DM) of dogs fed diets containing hydrolysates

Item	Diets ¹					SEM	P-value
	D_Shrimp	D_BSF-C	D_BSF-A	D_YM-C	D_YM-A		
Fecal output							
g/d, as is	135	134	140	138	126	5.6	0.434
g/d, DM basis	39.0	39.5	42.2	41.1	37.9	1.7	0.390
Number defecations/d	1.98	1.94	1.92	1.92	2.02	0.080	0.882
DM of feces, %	29.1	30.0	30.3	30.0	30.6	0.362	0.079
Fecal fermentation products							
pH	6.30	6.31	6.41	6.35	6.31	0.049	0.473
Ammonia-N	265	268	261	272	272	12.9	0.973
SCFA							
Total	845	885	869	863	843	22.1	0.651
Acetate	491	509	501	496	479	12.5	0.517
Propionate	237	250	248	241	236	8.2	0.647
Iso-butyrate	11.3	11.6	11.5	11.9	10.8	0.48	0.552
Butyrate	79.3	86.9	81.8	85.4	89.1	4.56	0.570
Iso-valerate	16.3	16.3	17.0	17.6	16.3	0.61	0.489
Valerate	4.99	6.25	5.68	7.00	8.26	0.874	0.108
Iso-caproate	3.74	3.25	3.55	3.87	3.48	0.302	0.638
Caproate	0.80 ^b	0.52 ^{a,b}	0.40 ^{a,b}	0.36 ^a	0.36 ^a	0.104	0.024

¹D_Shrimp, complete diet with shrimp hydrolysate; D_BSF-C, complete diet with black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; D_BSF-A, complete diet with black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; D_YM-C, complete diet with yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; D_YM-A, complete diet with yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L; a,b Values with different superscripts differ significantly ($P < 0.05$).

Table 6. Apparent total tract digestibility (ATTD, %) and metabolizable energy (ME) content of diets containing hydrolysates

Item	Diets ¹					SEM	P-value
	D_Shrimp	D_BSF-C	D_BSF-A	D_YM-C	D_YM-A		
ATTD, %							
Dry matter (DM)	81.1	80.7	79.4	79.3	81.3	0.75	0.191
Organic matter	85.9	85.7	84.7	84.7	85.9	0.56	0.298
Crude protein	81.9	81.1	80.0	79.2	81.8	0.84	0.148
Ether extract	95.5	95.5	95.0	95.1	95.3	0.20	0.292
NDF	67.8	67.3	64.2	64.6	66.9	1.32	0.211
Starch	99.6	99.5	99.6	99.3	99.6	0.08	0.161
Gross energy	87.1	86.7	85.7	85.7	86.7	0.50	0.201
ME, MJ/kg, DM	16.8	16.7	16.5	16.6	16.7	0.09	0.181

¹D_Shrimp, complete diet with shrimp hydrolysate; D_BSF-C, complete diet with black soldier fly (*Hermetia illucens*) hydrolyzed with Corolase PP; D_BSF-A, complete diet with black soldier fly (*H. illucens*) hydrolyzed with ALCALASE 2.5L; D_YM-C, complete diet with yellow mealworm (*Tenebrio molitor*) hydrolyzed with Corolase PP; D_YM-A, complete diet with yellow mealworm (*T. molitor*) hydrolyzed with ALCALASE 2.5L.

capture the full spectrum of antioxidant compounds present in the hydrolysate, as it measures the hydrophilic chain-breaking antioxidant capacity against peroxy radicals (Christodoulou et al., 2022). Antioxidant activity was observed in all hydrolysates evaluated in this study, agreeing with earlier results on shrimp (Latorres et al., 2018; Guilherme-Fernandes et al., 2024a) and YM and BSF hydrolysates (Zielińska et al., 2017; Teixeira et al., 2023). Insect hydrolysates produced with ALCALASE 2.5L showed a greater antioxidant capacity compared to hydrolysates produced with Corolase PP in both ORAC and ABTS methods, agreeing with the most frequent

production of smaller peptides with ALCALASE 2.5L rather than with Corolase PP as previously discussed. Additionally, the presence of aromatic AA in terminal chains, such as tyrosine, tryptophan, and phenylalanine, can neutralize free radicals, contributing to the antioxidant capacity of the hydrolysate (Romero-Garay et al., 2022). The evaluation of the peptides profile should be performed to fully understand the observed effects.

The antihypertensive activity of certain peptides is primarily attributed to their ability to inhibit the ACE, blocking the conversion of angiotensin I to angiotensin II, a potent

vasoconstrictor (Guerrero et al., 2012). Despite no apparent effect of the type of enzyme on antihypertensive activity being observed for BSF hydrolysates, YM-C required a lower concentration to inhibit the ACE activity than YM-A, showing a higher *in vitro* ACE inhibitory capacity. This result might be attributed to Corolase PP action that originates larger, hydrophobic peptides, with aromatic residues, earlier reported to improve the ACE inhibitory effectiveness. Conversely to our findings, Dai et al. (2013) reported lower required values for the ACE inhibition for YM hydrolysates obtained through enzymatic hydrolysis with ALCALASE 2.5L ($IC_{50} = 0.39$ mg/mL), while Rivero Pino et al. (2020) reported 0.27 mg/mL IC_{50} for YM hydrolysates obtained through enzymatic hydrolysis with trypsin. BSF hydrolysates did not show a significant *in vitro* antihypertensive activity (with IC_{50} values of 1 mg protein hydrolysate/ml), and no previous studies have shown the existence of antihypertensive peptides on BSF hydrolysates (Teixeira et al., 2023). Although Latorres (2018) reported the presence of peptides with ACE inhibitory effects in shrimp hydrolysates obtained through hydrolysis with Alcalase, in the current study, shrimp hydrolysate required double the concentration for 50% ACE inhibition compared with insect hydrolysates.

However, it must be emphasized that although both ABTS and ORAC assays are widely used to assess antioxidant potential *in vitro*, offering insight into a compound's free radical scavenging capacity, their correlation with *in vivo* antioxidant efficacy is limited due to differences in bioavailability, metabolism, and interaction with endogenous antioxidant systems. Nonetheless, they serve as useful preliminary screening tools as suggested by Huang and Prior (2005). Regarding the *in vitro* ACE-inhibition assay, several studies have demonstrated a correlation between potent *in vitro* ACE-inhibitory peptides and blood pressure-lowering effects in animal models as reviewed by Verduyck et al. (2005). Although *in vitro* ACE inhibition is an effective screening tool, the bioavailability and stability of peptides must be considered when predicting *in vivo* outcomes (Vermeirssen et al., 2004). Furthermore, it should be noted that in the present study, hydrolysates were added immediately before feeding and not included in the kibble, thus further studies should be performed to evaluate the effect of including insect hydrolysates before extrusion. Indeed, the information available is scarce and conflicting, with van Rooijen et al. (2013) suggesting that protein hydrolysates are more exposed to the Maillard reaction during extrusion than intact protein, and Rivero-Pino et al. (2020) reporting that peptide bioactivity, namely antioxidant and antihypertensive activity, can be kept or even enhanced during processing, depending on AA composition and time and temperature conditions.

Palatability

The two-bowl palatability test was performed to assess whether on top addition of insect hydrolysates could negatively impact food acceptance. No differences were observed between diets containing shrimp and insect hydrolysates, except for the YM-C diet, which presented a greater intake ratio. Although some caution should be taken when interpreting this result due to hydrolysates being added on top (Guilherme-Fernandes et al., 2024b), and to the limited number of animals used, the peptides and AA profiles might have played a role. Indeed, Alcalase action is known to release bitter hydrophobic peptides (Spellman et al., 2009) whereas

the broader enzymatic activity of Corolase contributes to reduced bitterness and improved palatability (Huang et al., 2021; Mirzapour-Kouhdasht et al., 2023). Earlier studies on the palatability of BSF and YM dried larvae for dogs have yielded conflicting results, with negative (Feng et al., 2020) and positive effects (Wei et al., 2024).

Feeding Trial

Slight adjustments of food daily allowance were made during the trial to keep an optimal BW and BCS. All hydrolysates were well accepted by dogs without negative effects being observed in food intake. Fecal output and score are important parameters for pet owners and are related to DM intake, dietary fiber, water-holding capacity of the dietary ingredients, nutrient digestibility, and gut microbiome, among other factors (Abd El-Wahab et al., 2021). All diets promoted formed and firm to softer stools, being the average number of defecations and fecal DM and output aligned with values expected for pet foods (Carcioli et al., 2008).

The availability of non-digestible proteins and fermentable carbohydrates reaching the colon, the gastrointestinal transit time, and the colon microbiota can affect the production and profile of fecal SCFA (Nery et al., 2012; Hall et al., 2013). No diet effect was observed on total SCFA production and profile, except for an increased concentration of fecal caproate with the control diet. Although this observed difference in caproate lacks biological relevance, it can be related to the type of peptides present in the shrimp hydrolysate and their interaction with the gut microbiome.

Fecal pH ranged from 6.3 to 6.4 across all diets, showing a desirable pH able to promote intestinal health (Wong et al., 2006), and being consistent with a lack of significant difference in fecal SCFA and ammonia concentrations between diets. This later absence of effects on ammonia concentration suggests that the amount of non-digested protein reaching the colon was similar among diets.

Although the evaluation of the impact of insect hydrolysates on stool quality and fecal metabolites is scarce, *in vivo* feeding trials with BSF meals have been conducted. Specifically, in a study with six Beagle dogs, the dietary inclusion of BSF meal produced well-formed and firm feces and kept stool consistency unaffected compared to a diet containing poultry meal (Abd El-Wahab et al., 2022). A change in the intestinal microbiome of dogs consuming a diet with 20% BSF meal was observed in another study, linked to decreased levels of fecal SCFA, with an increase in fecal pH (Jian et al., 2022).

ATTD and ME content were kept unaffected by the dietary inclusion of hydrolysates. All diets demonstrated CP and DM digestibility values around 80%, which are desirable values for nutrient digestibility in dry foods for dogs (Cargo-Froom et al., 2019). Several parameters can influence the digestibility of foods for dogs, including the source, quality and digestibility of ingredients, the amount of fiber in the food, the presence of anti-nutritional factors, the processing methods, and the interaction between ingredients (Cargo-Froom et al., 2019; Bos et al., 2023; Geary et al., 2023). It is not possible to compare current findings with literature because, to the best of author's knowledge, there are no published studies assessing the digestibility of foods for dogs containing insect hydrolysates. However, the *in vivo* digestibility of insect meals has been studied in the last few years, specifically assessing the inclusion of BSF meals for dogs as a main ingredient. Studies have shown that dogs can consume diets containing insect

meals, such as BSF larvae, house cricket, housefly larvae, lesser mealworm, and YM, without adverse effects on nutrient digestibility or general health (Bosch et al., 2014, 2016; Penazzi et al., 2021). As shown by Penazzi et al. (2021), in vitro and in vivo digestibility of diets containing BSF meals as the main protein source has shown similar protein digestibility compared with the venison meal diet, despite the crude fiber digestibility being lower in the insect-based diet, likely due to the greater chitin content. Moreover, Freel et al. (2021) reported no effects of different inclusion levels of BSF meals in the replacement of poultry byproduct meal and corn meal on ATTD of nutrients. While the current findings are promising, further research is needed to fully evaluate the long-term benefits from the dietary inclusion of insect hydrolysates, namely at greater percentages.

CONCLUSION

Overall, the present study has shown that BSF and YM hydrolysates present a high protein content with in vitro antioxidant and antihypertensive properties. Diet containing YM-C hydrolysate stood out for being more palatable and having greater antihypertensive activity when compared to the other studied hydrolysates. Insect hydrolysates were well accepted by dogs when included at a 3% (w/w) inclusion level without compromising diet intake and digestibility and fecal characteristics and metabolites. However, the lack of an evaluation of the peptide profile of hydrolysates limits the mechanistic understanding of their bioactive properties. Moreover, conducting long-term trials to explore in vivo functional effects, such as antihypertensive activity, modulation of the gut microbiome and immune function, and chronic disease prevention represents an important direction for future research. As extrusion may alter palatability, peptide structure and bioactivity, future studies should validate the functional properties of insect hydrolysates when included in complete diets produced under commercial conditions, to ensure translational relevance and applicability to the pet food industry.

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Author Contributions

D. Leal (Formal analysis, Investigation, Writing - original draft), S. Borges (Formal analysis, Investigation, Writing - review & editing), A. Almeida (Funding acquisition, Project administration, Writing - review & editing), M. Pintado (Funding acquisition, Project administration, Supervision, Writing - review & editing), António Fonseca (Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing), and Ana Rita Cabrita (Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing - review & editing).

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Conflict of Interest statement

Authors report no potential conflict of interest.

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